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Functional Hallmarks of healthy Macrophage Responses: Their Regulatory Basis and Disease Relevance

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Abstract

Macrophages are first responders for the immune system. In this role, they have both effector functions for neutralizing pathogens and sentinel functions for alerting other immune cells of diverse pathologic threats, thereby initiating and coordinating a multipronged immune response. Macrophages are distributed throughout the body: they circulate in the blood, line the mucosal membranes, reside within organs, and survey the connective tissue. Several reviews have summarized their diverse roles in different physiological scenarios and in the initiation or amplification of different pathologies. In this review, we propose that both the effector and the sentinel functions of healthy macrophages rely on three hallmark properties: Response Specificity, Context Dependence, and Stimulus Memory. When these hallmark properties are diminished, the macrophage's biological functions are impaired. That in turn results in increased risk for immune dysregulation, manifested by immune deficiency or auto-immunity. We review the evidence and the molecular mechanisms supporting these hallmark properties.

Keywords

innate immunity; macrophages; sentinel cells; stimulus specificity; polarization; epigenetic memory; NF κ B; IRF; MAPK; Interferon; chromatin

Preface

Macrophages were first described in the late 19th century, when Ilya Mechnikov looked into his microscope and noted a striking observation: 'white corpuscles' moved to surround a small splinter embedded in a starfish larva (1). This first description of the cells now known as macrophages launched investigations of innate immunity.

Macrophages are first responders, capable of detecting tissue injury and pathogen threats. They respond to such threats with two broadly defined functions: 1) they have potent

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anti-pathogen effector functions as their name implies, and 2) they also have potent sentinel functions to call up and direct innate and adaptive immune cells. As effectors, macrophages phagocytose pathogens, upregulate antimicrobial peptides, or trigger cell death to limit intracellular pathogens (Figure 1a). As sentinels, macrophages initiate and coordinate local or systemic immune activation by secreting cytokines, chemokines and growth factors, and presenting antigen to adaptive immune cells (2).

As first responders, macrophages are strategically placed. Almost every tissue in the body is populated with macrophages at a remarkably consistent density of $\sim 500\text{--}1000/\text{mm}^3$ (3–5). Tissue resident alveolar macrophages, peritoneal macrophages, and Kupffer cells of the liver sense airborne pathogens or those from the digestive tract. In addition, bone-marrow-derived monocytes circulate the blood and readily extravasate into tissues upon sensing chemoattractants secreted by the very first responders. Upon sensing a pathogen-associated molecular pattern (PAMP) or damage-associated molecular pattern (DAMP) their differentiation into macrophages is accelerated, rapidly contributing effector and sentinel functions at the site of infection or injury.

Macrophages, despite having disparate ontogenies and a wide variety of physiological roles, share certain functional characteristics. These characteristics are less defined by developmental origin or their steady state (as seen in diverse epigenomic or transcriptomic profiles (6)), but rather by the manner in which they respond to immune threats. Here we propose three functional hallmarks that define healthy macrophages (Figure 1b): 1) Response Specificity – the capacity to mount threat-specific responses. 2) Context Dependence – the capacity to adapt immune threat-specific responses to the microenvironmental cytokine milieu. 3) Stimulus Memory – the capacity to record prior exposure to stimuli and adapt subsequent responses to immune threats.

Does the misregulation of these functional hallmarks contribute to risk for disease? The first hallmark of Response Specificity is a measure of the distribution overlap in sentinel responses to different ligands; single cell response distributions with abnormal spread may result in improper immune sequelae. Autoimmune diseases, where symptoms are often sporadic with unknown triggers, may involve the loss of healthy Response Specificity. Second, Context Dependence of macrophage responses are linked to diseases of aberrant cytokine conditioning. As opposed to the genetic changes that are often risk factors for autoimmune disease, infectious disease complications or inflammatory diseases like cancer are affected by microenvironment states such as aging and obesity, and may reflect poor regulation of context-dependent responses. Third, diseases of Memory may result when the primary stimulus improperly alters the epigenome, resulting in hyper- or hypo-inflammatory disorders upon a second encounter, rather than inducing innate immune memory that results in benefits against subsequent infectious agents. It is of great interest to delineate how the loss of the functional macrophage hallmarks may result in disease, prompting a focus on understanding of the molecular mechanisms that give rise to each functional hallmark.

The mechanisms underlying signaling and epigenome regulation of macrophage responses have been closely studied and reviewed (20–23). However, it has been less obvious how these molecularly-detailed biochemical and biophysical mechanisms give rise to

the immunological functions of macrophages. Recent studies quantifying the stimulus-specificity of signaling dynamics (24), on feedforward and feedback mechanisms (25, 26), or on context- or exposure-dependent epigenomic changes (27, 28), have further elucidated these connections. Furthermore, new technology such as reliable live-cell microscopy, cost-effective scRNAseq and multi-dimensional flow cytometry, and algorithmic advances in analysis of the data, have allowed us to probe and measure these hallmarks accurately. In light of these advances, in this review, we place existing and recent mechanistic knowledge into the framework of these three functional hallmarks that characterize healthy macrophage identity.

1. Response Specificity

Macrophages express dozens of receptors and are capable of sensing hundreds of PAMPs, DAMPs, and cytokine ligands. Immune response genes are not expressed constitutively because they are in fact detrimental to the host. Hence, they must be deployed on an “only-as-needed” basis. Given that pathogens differ widely in their biology, different immune defenses are required to effectively counteract them. The “only-as-needed” rationale argues that healthy immune sentinel cell responses should be highly specific to the immune threat.

It is now appreciated that macrophages respond to ligands with stimulus-specific signaling profiles and stimulus-specific gene expression programs (9, 29, 30). Not only ligand, but also ligand dose, and ligand exposure dynamics and duration generate distinct responses (31–34). Yet, early studies of macrophages reported a common core response, emphasizing the common phagocytic and antigen-presenting functions of macrophages (35). Only the resulting adaptive immune responses were thought to provide specificity to the immune response. However, later studies revealed that macrophages, dendritic cells, and fibroblasts (36) produce gene expression programs that are in fact stimulus-specific (9, 37), and began to address the molecular mechanisms that allow for this Response Specificity.

Macrophages perform as individuals in their function as sentries; each has the capacity to sense molecular patterns and activate signaling pathways to generate a response, and the immune response relies on the response of the individual. Yet, individual macrophages are not identical, being subject to molecular stochasticity that affects gene expression (and hence the abundances of pathogen sensors and signaling proteins) and the cells’ spatial organization. Furthermore, macrophages within tissue are comprised of distinct cellular subsets (38), including subsets with distinct developmental origins (39). The resulting cell-to-cell heterogeneity may affect stimulus-response programs, and thus the Response Specificity ascribed to the population. Therefore, quantitative studies of Responses Specificity must involve measurements at single cell resolution.

1.1. Immune response signaling network

Response Specificity relies on molecular components and pathways that are activated stimulus-specifically. Given the large number of immune activating stimuli but a limited number of signaling factors, two principles have emerged that help to explain the stimulus-specific regulation of the molecular network: combinatorial and dynamic control of signaling pathways. These principles describe that stimulus-specific activation of immune

response genes is dependent on 1) different stimuli activating different combinations of signaling regulators, and 2) different stimuli activating the same regulator but with different dynamic patterns of activity.

Specific responses are initiated by how cells sense ligands. Human macrophages sense PAMP and DAMP ligands with 10 Toll-like receptors (TLRs) (40), that reside in the plasma membrane and survey extra-cellular and endosomal environment, 22 cytosolic NOD-like receptors (NLRs) (41), 3 RIG-I-like-receptors (RLRs) (42), and DNA sensor cGAS (43, 44). Immune activating cytokines such as interferons, IL1, TNF and other TNF super family members are sensed by their cognate receptors in the plasma membrane. To elicit stimulus-specific responses the ligand-receptor interaction must be specific based on the specificity of the molecular interaction. Indeed, even low complexity PAMPs are distinguished by different TLR family members, and the biophysical basis for these this specificity has been studied (45). However, functional discrimination of ligands may also involve other mechanisms such as localization (46) or kinetic proof-reading, as for example in the case of RIG-I (47).

Despite the impressive number of pathogen sensors, the number of pathogens and potential PAMPs and DAMPs is even greater. Many receptors have been shown to mediate sensing of distinct ligands. TLR4, for example, the known endotoxin (LPS) sensor, also mediates sensing of DAMPs, such as HMGB1 (48), serum amyloid A (SAA) (49), and MRP8/MRP14 (50). The range of interactions may be expanded by soluble or membrane-bound co-factors such as CD14 (51). However, two ligands being recognized by the same receptor does not necessarily mean that they produce the same response. Ligand-receptor interactions are governed by specific kinetics of signaling adaptor recruitment, receptor internalization, and ligand degradation, which may produce not only stimulus-specific amplitudes of signaling but stimulus-specific signaling dynamics. Such dynamic coding mechanisms also allows for cells to mount dose-specific responses for the same stimulus via the same receptor.

Similar considerations govern how receptors activate downstream signaling pathways, which are mediated by a small number of primary signaling adaptors MyD88, TRIF, TRAF2/6, MAVS, STING, and ASC (52). In fact, all TLRs, except TLR3 utilize MyD88, as does the IL1 receptor. TRIF is engaged by TLR3 and TLR4. TRAFs are engaged by TNFR family members, ASC by NLRs, and STING by cGAS (53). However, specificity is possible because the relative strengths and dynamics of adaptor recruitment and signaling pathway activation may differ between receptors. For example, in response to lipopolysaccharide (LPS), TLR4 signals at the plasma membrane and initiates the oligomerization of the adapter Myd88, or can be internalized to signal through the endosome, where it interacts with a different adapter TRIF to initiate TRIF-dependent signaling. LPS dose-response specificity is controlled at the adapter level by the different oligomerization dynamics of Myd88 and TRIF (54).

Adapters and associated ubiquitin chains ultimately activate a limited set of kinase-transcription factor modules. While interferons activate the JAK-STAT pathways without a dedicated signaling adaptor, four primary immune response signaling pathways may be mapped onto these adaptors: IRF3 is activated by TRIF, MAVS and STING adaptors,

MAPKp38 is strongly activated by MyD88, but NF κ B and JNK/ERK pathways are activated by all adapters (in the case of ASC via the IL1b feedback loop) (53). Therefore, if Response Specificity solely relied on combinatorial coding only three patterns could be observed: NF κ B + JNK/ERK (e.g. in response to TNF), NF κ B + JNK/ERK + IRF3 (e.g. in response to poly(I:C)), NF κ B + JNK/ERK + MAPKp38 (e.g. in response to Pam3CSK4), and NF κ B + JNK/ERK + MAPKp38 + IRF3 (e.g. in response to LPS). However, stimulus-specific dynamic control of these pathways may allow for additional specificity (see next section).

Given that Response Specificity is not merely a function of biophysical interaction specificities that are genetically encoded in protein structures, but also of signaling dynamics in amplitude and time, it is subject to differences in the expression levels and localization of signal transducers. It is commonly appreciated that the PRR repertoire that macrophages express determines their responsiveness. The above discussion suggests that the Response Specificity of macrophages is diminished by cell-to-cell heterogeneity in the expression of PRRs, and other key signaling regulators (e.g. adaptors, kinases, etc), also. In response to LPS, the primary driver of the heterogeneity of NF κ B responses was in fact found to be, not the TLR4 abundance, but the maturation time of the endosome, which determines the duration of TRIF signaling (54).

1.2. Immune response transcription factors

Response Specificity, the capacity to mount threat-specific responses, has been studied at the level of transcription factor activities, given the availability of quantitative assays. Biochemical studies performed in fibroblasts in the 2000's elucidated both the temporal and combinatorial regulation of kinase-transcription factor activities mediating stimulus-specific mean responses (36, 55, 56) (Figure 2a). For example, NF κ B activation was shown to be persistent in response to LPS, but oscillatory in response to TNF, mediated by negative feedback regulation from I κ B α (24, 57). Similarly, JNK activation showed two distinguishable phases of activity (58). Combinatorially, while the kinase-TF modules IKK-NF κ B and JNK-AP1 are ubiquitously, adapter-specific activation of TBK1-IRF3 by TRIF and MAPKp38 by Myd88 further contribute to Response Specificity of TF activity (9, 59, 60).

However, with the advent of single cell assays, the cell-to-cell heterogeneity of macrophage populations has come into focus. Studying Response Specificity must therefore not only address the distinction of mean responses with population level (bulk) assays, but about how well the distributions of responses across single cells may be distinguished. Not until the 2010's was appropriate single cell technology and an analytical framework (based on information theory) developed to allow insight into the distinction of single-cell response distributions. Measuring NF κ B activity levels at a single timepoint, TNF dose-response specificity was found to be low; while 12 different TNF doses spanning 4 orders of magnitude were measured cells were barely able to reliably distinguish the presence of absence of TNF (61). Subsequent studies showed that the temporal dynamics of signaling activity enabled by live-cell microscopy on fluorescently tagged proteins provides information for stimulus-distinction (24, 32, 62–64). Mechanisms of positive feedback from RelA were identified as one component enabling LPS dose-specificity (65). Furthermore,

across a range of doses and an array of immune stimuli targeting both TLRs and cytokine receptors, six NF κ B dynamical features, termed signaling codons, were shown to be key to maximally facilitating specificity to ligand dose and ligand identity (24). All six NF κ B signaling codons are determined by precise modulation of IKK activity over time: the I κ B α negative feedback loop serves to amplify small differences in IKK activity (low vs medium-low) by converting them into oscillatory vs non-oscillatory NF κ B trajectories. However, which sources of molecular noise are the primary drivers of the heterogeneous deployment of each NF κ B signaling codon remains unclear, and how such responses are modulated by microenvironmental or polarizing cytokines is still an active area of investigation.

Single-cell studies have also begun to quantify to what extent the combinatorial activation of signaling pathways is a contributor to Response Specificity. Simultaneous measurement of NF κ B and MAPKp38 in single cell macrophages at a single timepoint revealed that dose-response curves for each pathway were distinct, with MAPKp38 being digitally activated above a ligand concentration threshold (32). Therefore the combination of NF κ B and MAPK with differential dose response curves may have a larger overall dose-discrimination capacity than either pathway alone. Another study that measured single cell temporal dynamics for both NF κ B and JNK indicated that the two pathways combined were biologically informative and reflective of different levels of threat from pathogenic vs non-pathogenic microbes (62). The development of kinase translocation reporters for MAPK ERK, p38, JNK activity may allow evaluation of the combinatorial and temporal control of these signal transducers in single cells (58). Less is known at the single cell level about the combinatorial and dynamic control and heterogeneity of IRF signaling.

1.3 Immune response gene expression

Downstream of signaling pathways, Response Specificity is evident and may be measured at the level of immune response gene expression programs. Bulk transcriptomic studies identified sets of genes controlled by stimulus-specific combinatorial activation of signaling pathway activity (9). These gene sets could be mapped to the immune response transcription factors, identifying sets regulated by single TFs NF κ B, IRF, or AP1, but also those that required two pathways, such as a cytokine set whose transcriptional activation is mediated by NF κ B and whose post-transcriptional regulation of mRNA half-life is mediated by MAPKp38. *Ifnb1* and *Ccl5* are other well-known examples of genes requiring the combinatorial activation of NF κ B and IRF (66, 67).

Many previous studies relied on clustering algorithms of transcriptomic data to identify patterns of gene expression. These approaches readily identified a handful of distinct patterns when data from multiple ligands is available (9, 37), but closer inspection reveals many more patterns of expression, some of which may only be exhibited by a single gene (66, 68). Thus analytical approaches that have true single gene resolution are critical. For example, the duration of NF κ B activity could be shown to be decoded gene-specifically using a mechanistic modeling approach with differential equations rather than a statistical evaluation that requires considerations of sets of genes; for each gene the contribution of duration decoding by mRNA half-life or chromatin mechanisms could be quantified (11). A similar mechanistic modeling approach has been developed to elucidate the combinatorial

transcription factor control of gene expression at single gene resolution (60) but its implementation requires a very large amount of data from different conditions.

While prior gene expression studies at the population showed that transcriptional responses are ligand-specific and could elucidate regulatory mechanisms, the quantification Response Specificity requires single cell resolution data, as cell-to-cell heterogeneity affects this quantity. Interestingly, the cell-to-cell variability present in signaling pathway dynamics and activation levels may, in principle, be either buffered or amplified by the chromatin-associated or post-transcriptional regulatory mechanisms controlling the expression of each gene. Technological developments may be required to link signaling to gene expression in the same cell, such as advances in microfluidics, image analysis, smFISH or scRNAseq (Figure 2b) (69–71). In one study in the macrophage RAW246.7 cells, after measuring NF κ B signaling dynamics in response to LPS and then profiling the transcriptome of each cell at the end point, it was found that the expression of some cytokine and feedback regulator genes were correlated to the cell's NF κ B dynamics (72). In addition, certain pairs of genes possibly controlled by the same enhancer elements maintained correlated expression levels across single cells. However, a key limitation of some single cell assays is the high degree of technical noise. Further work in profiling transcriptomic Response Specificity across multiple ligands and doses are needed to understand the extent to which signaling dynamics are decoded stimulus-specifically. As with studies of signaling dynamics in sentinel cells, both statistical analysis and mechanistic modeling may prove insightful in elucidating mechanisms and sources of biological noise in single cell gene regulation.

1.4. Cytokine loops

One product of the primary signaling response is the secretion of immune response cytokines that may then be sensed in an autocrine or paracrine manner. They may function as feedback and feedforward loops to contribute to Response Specificity.

The activation of the IRF pathway by PAMPs such as LPS and poly(I:C) induce the secretion of interferon- β (IFN β), which acts in a feedforward loop to produce ISGF3 that reinforces the IRF3-driven gene expression programs in primary response cells, and produces an almost equivalent interferon-response program neighboring bystander cells (73). A single-cell study in dendritic cells responding to LPS showed that early paracrine secretion of IFN β in just a handful of cells was important for antiviral gene expression in the population; at later timepoints, *Ifnar* and *Stat1*-dependent IFN β paracrine signaling downregulated inflammatory genes not uniformly, but in a fraction of the cell population (74). This presence or absence of negative feedback from Type I interferons was shown to be biologically important for distinguishing Gram-negative versus Gram-positive bacteria in BMDM responses. Bacterial class-specific production of key cytokines such as CXCL1 and TNF was diminished in either IFNAR knockouts, or when IFN β was exogenously supplied (25, 75).

Like interferons, TNF production also may further amplify or curtail Response Specificity. Single cell studies of NF κ B signaling dynamics revealed that in the presence of TNFRII, a soluble TNF inhibitor, the responses to low dose CpG or LPS stimulation became less variable by reducing the oscillatory component from response trajectories (24). In another

study, blocking TNF autocrine signaling decreased the heterogeneity of NF κ B signaling profiles in response to LPS, suggesting that cell-to-cell variability of signaling was in part affected by the heterogeneity of cellular secretion of TNF (72).

In the case of NLR signaling, the secretion of IL1 β and IL18 is in fact critical for the activation of NF κ B and MAPK pathways (76, 77) – these are the pathways that allow for gene expression responses in both primary responders and bystanders. Thus, the production, secretion, and responses to soluble cytokines, and the single cell heterogeneity of these processes, may be a feature that can either expand or restrict sentinel ability to discriminate dose or identity of a pathogen or DAMP ligand.

1.5. Diseases of impaired Response Specificity

By proposing that Response Specificity is a property of healthy macrophage function, we suggest that impaired Response Specificity contributes to disease. Healthy Response Specificity may be characterized by particular response distributions to each immune threat, and both increases or decreases in the heterogeneity may result in disease (Figure 2c). As such, in these diseases, the behavior of outliers is critical. Rheumatoid arthritis, lupus, multiple sclerosis, and Sjogren's Syndrome all have remitting and relapsing characteristics (78–80). The intermittent nature in the presentation of multiple autoimmune diseases hints that low probability outlier events may underlie its etiology.

Indeed, both aberrant TNF and IFN β regulation have been implicated as opposing sides of different autoimmune diseases (81). Excessive IFN β production from dendritic cells was postulated to be an initiator of the autoimmune disease systemic lupus erythematosus (SLE) (82). As IFN β has both feedforward and negative feedback functions on neighboring cells, the improper production of IFN β from even a subset of cells may have significant consequences on Response Specificity. A recent single-cell study more directly couched the autoimmune disease Sjogren's Syndrome as involving loss of Response Specificity. Loss of the NF κ B negative feedback regulator *Nkbia* diminished the distinguishability of macrophage responses to TNF vs poly(I:C), interestingly through the increased expression of IRF target genes in a fraction of TNF-stimulated cells (24). TNF-induced IFN β production through IRF1 has also been implicated in rheumatoid arthritis, which could be corrected through JAK inhibitor drugs (83). Thus, the misregulation of the TNF vs. IFN β axes in autoimmune disease may provide clues into how to correct or control loss of Response Specificity (84).

2. Context Dependence

Macrophages populate all organ systems and are therefore exposed to different cytokine microenvironments that provide instructions for tailoring the function to the local physiological state. As a result, macrophage responses and functions have evolved to be highly adaptable. Interestingly, macrophages residing in the same organ system may have different developmental origins: a portion of the resident population are longer-lived derivatives of the yolk sac or fetal liver, while another portion are monocyte-derived and migrate to the tissue from the blood stream (85). However, their immunological functionality is a product of both ontogeny and cytokine cues of the local environment, such that

all tissue-resident macrophages within an organ adopt similar functions (86). Polarizing cytokines in the microenvironment can further shift the response repertoire of macrophage to allow them to carry out more specialized roles. This tuning of function is important in normal physiology in the contexts of inflammation, injury, or repair.

We thus propose the second hallmark of healthy macrophage responses is Context Dependence. Context Dependence allows for the canalization of macrophage functions, in which both signaling networks and epigenetic states are tuned to the current microenvironment to promote specialized functions while the tuning cytokine is present (Figure 3a). Originally, for monocyte-derived macrophages, a simplified paradigm of Context Dependence existed within the framework of M1 and M2 macrophage polarization (87). M1 macrophages are canonically anti-microbial, producing pro-inflammatory cytokines, and upregulating their phagocytosis ability; M2 macrophages are canonically associated with repair, producing anti-inflammatory cytokines, or growth factors. While a much more complex topology of macrophages activation states is now known to exist, pro- and anti-inflammatory M1-IFN γ and M2-IL4 states generally represent opposing extremes of macrophage context-dependent functions (23, 88).

2.1. Signaling mechanisms

What are molecular mechanisms that allow for Context Dependence? Both signaling crosstalk and epigenetic mechanisms allow macrophages to respond in relation to their microenvironment context. Generally, signaling networks that encode ligand-specific responses may be altered by polarizing cytokines that change the availability of signaling components (89). Such components may include receptors, adapters, transcription factors, feedback regulators, and even regulators of core machineries responsible for protein synthesis or decay.

Microenvironmental IFN can affect PAMP- or DAMP-induced signaling by altering expression levels of signaling pathway components: Type I interferon IFN α upregulated the expression of TLRs TLR3, TLR4, and TLR7 (90), and Type II interferon IFN γ upregulated transcription factor IRF1 that augments TLR-dependent IFN signaling (91). Modulation of feedback regulator activity is also critical: IFN β increased NF κ B activity by reducing the translation of I κ B α , and in the late-phase, through increased IKK activity via expression of the viral RNA sensor RIG-I (92). IFN γ was shown to elevate NF κ B activity through increased expression of proteasomal cap components that degrades the feedback inhibitors of NF κ B, I κ B α and I κ B ϵ (92, 93). Another cross-regulatory feedback protein has roles as a negative regulator of NF κ B. NLRC5, a NOD-like receptor family member, is induced by IFN γ (94), and decreases NF κ B activity via inhibition of IKK α /IKK β phosphorylation. NLRC5 also negatively regulates type I interferon signaling at the receptor level via inhibition of RLR-mediated type I interferon responses (95) (Figure 3b).

Within the interferon pathway itself, polarizing cytokines induce negative regulators such as SOCS proteins that may also both enhance or suppress PAMP or DAMP-responses. Low dose IFN α increases baseline STAT2 and IRF9 expression without strongly activating the negative feedback regulators SOCS1, SOCS3, and USP18, thereby hypersensitizing cells to further interferon stimulation (26). On the other hand, higher levels of type I interferon

may increase the expression of these negative regulators: recruitment of USP18 to the type I interferon receptor IFNAR desensitizes cells to any additional IFN-activating stimuli (96, 97), while increased SOCS1 expression suppresses responses to pathogens that activate interferon-pathway signaling like LPS (98). IFN γ was reported to upregulate the expression of SOCS3, which inhibits STAT3 activity and promote M1 macrophage activation (99). In contrast, IL4 was reported to upregulate SOCS1 but not SOCS3, thereby inhibiting STAT1 transcription factors and promoting the M2 macrophage phenotype (100). The strength of crosstalk is modulated by dose and duration of the polarizing cytokine cue, but may also be subject to other cell properties like cell-cycle phase (101).

2.2. Epigenetic mechanisms

Epigenetic mechanisms constitute a subsequent layer of control critical to context-dependent canalization of macrophage functions. Importantly, unlike signaling mechanisms, epigenetic control results in gene-specific rather than pathway-specific alterations. IFN γ -M1 and IL4-M2 polarization are associated with chromatin alterations at STAT1/IRF1 and STAT6 enriched genomic regions, respectively (102). Interestingly, macrophages exposed to multiple tuning cytokine signals simultaneously, such as the opposing polarization cytokines IFN γ and IL4, as might occur in tissue or bloodstream microenvironments, did not show extensive antagonism at the level of signaling pathway activation; instead cross-talk occurred more prominently at the gene regulatory level through gene-specific binding of STAT1 or STAT6 (103).

Interferon priming can potentiate some subsets of genes, while repressing others. In human macrophages, type II interferon IFN γ synergistically enhanced TLR-induced transcription at inflammatory genes TNF, IL6, and IL12B by recruiting STAT1 to enhancers and promoters to increase chromatin accessibility and prime genes without itself inducing transcription (91). Type I interferon IFN α/β also impacts chromatin. In cooperation with TNF, IFN α generated increased chromatin accessibility at specific sites to potentiate the pro-inflammatory effect of LPS (104). Monocytes stimulated with LPS from patients with systemic lupus erythematosus (SLE) displayed epigenomic similarity to those primed *in vitro* with IFN α and TNF, suggesting that IFN α exposure *in vivo* alters chromatin to contribute to inflammatory symptoms of SLE (104, 105). In addition to enhancing the expression of inflammatory genes, IFN γ was also found to repress M2-like genes by disassembling MAF and LDTFs PU.1 and C/EBP β bound at select enhancers (28) (Figure 3c). At other genomic locations, IFN γ also suppressed enhancers associated with STAT3 (106). Notably, the genes at MAF enhancers were also repressed in macrophages from rheumatoid arthritis patients, suggesting that both potentiation and repression at the chromatin level by IFN γ priming may play roles in autoimmunity.

2.3. Specialized physiological functions via polarization

By affecting signaling and epigenetic mechanisms, contextual cytokines may alter macrophage functions to enhance protection of the host organism from pathogen threats or minimize collateral damage of inflammation. The presence of both type I and type II interferon in the microenvironment may prime macrophages for an LPS challenge, enhancing the expression of innate immune genes and cytokines (107). Polarization can also

serve the function of resolving or more carefully regulating inflammatory processes. IFN γ priming was found to repress a portion of LPS-inducible genes, resulting in a reduction in the recruitment of neutrophils to the inflammatory site (108). These changes in the production of chemokines by macrophages can ultimately rewrite the script of systemic immune activation.

In addition to altering the level of expression of inflammatory gene programs, microenvironment context can also tune cell-to-cell heterogeneity, which is a determinant of Response Specificity. A single cell study measured ‘propagation of variance’ in gene expression within gene regulatory networks in human macrophages, and found that IFN γ +TNF, IL4, and IL10 cytokine environments each generated distinct changes in biochemical parameters within the signaling network, altering patterns of cellular heterogeneity (109). For example, IL10 signaling increased the phosphorylation and nuclear localization of ATF2, which in turn tuned the variability of ATF2 target gene expression. The adaptation of macrophage heterogeneity to cytokine microenvironments may be beneficial as a “bet-hedging” strategy that leverages an altered distribution in single cell responses to immune stimuli (109).

The microenvironment of different organ systems constitute different types of Context Dependence of macrophage function (110, 111). Tissue-specific environments selectively activate transcription factors that collaborate with PU.1 to establish distinct sets of enhancers and super-enhancer in the resident macrophage population (112, 113). This tissue-specific functional polarization of macrophages is reversible and held in place by transcription factors induced by the microenvironment. For the inflammatory potential of the peritoneum, retinoic acid (Vitamin A) from local tissues polarized peritoneal macrophages by inducing GATA6 (114). Interestingly, the expression of this peritoneal macrophage-specific gene was not required for peritoneal macrophage development and was decreased by depletion of Vitamin A signal, pointing to the role of GATA6 as a peritoneal polarization gene rather than a lineage-determining transcription factor. Thus, through constant surveillance of the contextual environment within the tissue, tissue-resident macrophages may be able to dynamically adjust their response potential (114).

Furthermore, the presence of polarizing cytokine contexts can also alter the macrophage’s metabolism. IFN β has been shown to mediate metabolic reprogramming. For example, exposing BMDMs to live *M. tuberculosis* restrained glycolysis and mitochondrial stress, a phenotype recapitulated by IFN β stimulation alone and abrogated in IFNAR knockout mice. This metabolic form of priming was hypothesized to affect macrophage responses to bacteria by decreasing macrophage energy metabolism during mycobacterial infection, though whether this phenomenon was beneficial or detrimental remains unclear (115). Thus, even in the absence of acute infection, the presence of type I interferons in the environmental milieu have marked effects on signaling nodes central to pathogen or cytokine responses (116).

2.4. Altered functions in disease contexts

While Context Dependence is a feature of healthy macrophage function, dysregulated microenvironments may also have detrimental effects on macrophage responses. Indeed,

several chronic inflammatory diseases harbor aberrant cytokine microenvironments, which alter macrophage responses. Conditions common to human health include aging, which can involve an inflammatory component and has been termed inflammaging (117), or obesity, which is a disease of chronic metabolic inflammation (118, 119).

Both age and obesity have been linked to poorly regulated immune responses (120). For instance, a comparison of macrophages from old and young mice suggested that age impairs macrophage polarization, with significant decreases in M1 and M2 marker genes after exposure to polarizing ligands (121). Failure of macrophages to appropriately polarize may lead to less effective context-dependent immune responses. Similarly inflammation-associated aging may affect macrophage context-dependent responses through metabolic, signaling, and epigenetic mechanisms (122). This chronic low-grade inflammation has been linked to immunosenescence and is marked by increases in circulating pro-inflammatory cytokines such as TNF and IL6 (123–126). Furthermore, the Context Dependence of responses in aged macrophages is tissue specific. Within aging skeletal tissues, macrophages adopt a more M2-like phenotype (127), but the pro-inflammatory cytokine environment is hypothesized to produce age-associated pro-inflammatory M2-like macrophages (122). Further work may delineate the effect of age on the ligand responses of macrophages from different tissues and under a variety of polarization conditions.

Obesity is a second disease-associated inflammatory context that affects macrophage responses. Obesity causes inflammation of the adipose tissue, due to the release of IFN γ by natural killer cells (128). In lean mice, tissue-resident adipose macrophages retained an anti-inflammatory M2-like state. However, under a high fat diet, macrophages in the adipose tissue accumulate and adopt an M1-like pro-inflammatory phenotype marked by increased TNF and IL6 expression (129). Interestingly, macrophages in obese IFN γ knockout mice shifted towards an M2 phenotype, and organisms displayed improvements in insulin sensitivity (130). It remains to be seen by what mechanisms the polarized states of macrophages due to metabolic inflammation influences the effectiveness of their innate immune responses to immune threats.

3. Stimulus Memory

Stimulus Memory refers to a longer-term property of macrophages to record past exposures within its epigenetic state. The identity of differentiated cells is defined by the epigenomic enhancer landscape, which is held in place by the stable expression of a set of cell-type-specific transcription factors (131, 132). This category of transcription factors, termed lineage-determining transcription factors (LDTFs), are pioneer factors (133, 134) that have structural elements that enable them to bind to nucleosomal DNA and adjust the enhancer landscape during development (135). While the epigenetic landscape determines cell identity, epigenome plasticity allows differentiated cells to adapt their functions to environmental cues (Figure 4a).

The third functional hallmark of macrophages responses, Stimulus Memory, concerns the malleability of the developmentally established epigenetic landscape, whereby signal-dependent transcription factors (SDTFs) are able to trigger the formation of new enhancers.

When those stimulus-specific changes to the chromatin epigenome are not reversed when the SDTF activity ceases, epigenetic memory of the prior exposure event is formed. Stimulus Memory is thus distinguished from Context Dependence, in that that the inducing signal need not persist, and that signal is often generated by PAMPs and DAMPs rather than polarizing cytokines. Thus, Stimulus Memory stores marks of previous exposure to influence sentinel cell responses to future stimuli.

3.1. Transcription factors, nucleosome remodelers, metabolites

Several classes of molecules in the nucleus mediate Stimulus Memory: transcription factors, nucleosome remodelers, metabolites. For stimulus-induced epigenetic programming to occur, SDTFs, which bind to DNA, must be activated (136). SDTFs like AP1, NF κ B and IRFs are activated stimulus-specifically by immune threats, but unlike LDTFs, they are not cell-type specific. Because the combinations and dynamics of SDTF activities are stimulus-specific, epigenetic memory may also be stimulus specific. Patterns of SDTF-DNA binding were shown to enable the stimulus-specific formation of *de novo* enhancers marked by H3K4me1 deposition, a covalent modification to the chromatin landscape (136). More recently, the mechanisms by which the temporal dynamics of SDTF activity control *de novo* enhancer formation have also been elucidated. In macrophages, non-oscillatory NF κ B activity allowed the necessary continuous nuclear residence time for nucleosome eviction and eventual H3K4me1 deposition (27). Because cytokine and viral PAMPs induced oscillatory NF κ B activity, while bacterial PAMPs induced non-oscillatory activity (24), the stimulus-specific dynamics of transcription factor activity determined the extent of epigenomic enhancer formation (27) (Figure 4b).

Because SDTFs like NF κ B and IRF are not LDTFs, it seemed unlikely that they could impart long-term changes to the epigenome. However, biochemical and cryo-electron microscopy studies suggested that SDTFs like NF κ B could bind to nucleosomal DNA and potentially displace histone H1 (137, 138). The spontaneous unwrapping and rewinding 'breathing' of DNA around the histone core octamer suggests that even SDTFs could invade the nucleosomal DNA-histone octamer complex (139, 140), by preventing rewinding of the SDTF-bound sections of DNA (141). However, the rates of spontaneous rewinding were rapid enough that NF κ B could only bind to its cognate motif when it was positioned at the edge of the nucleosome, and not its interior. Thus, while stimulus-specific epigenetic memory is mediated by the activation of SDTFs, only a specific nucleosomes may be targeted, and cooperative mechanisms from other proteins may be required (142).

LDTFs are distinct in each cell type, and their binding to chromatin regions contributes to establishing the cell type's gene expression potential. In macrophages, PU.1 and C/EBP β are the LDTFs that are known to promote nucleosome-free regions and establish macrophage identity (143). LDTFs, being pioneer factors, have the ability to directly perturb nucleosome structure (144) and thus cooperatively hold open chromatin regions for gene expression. LDTFs mark potential chromatin regions for the subsequent binding of non-pioneer factors and other nucleosome remodeling complexes, resulting in the deposition of histone modifications that produce epigenetic memory. In macrophages, the LDTF PU.1 colocalized with areas of SDTF binding and H3K4me1 deposition upon stimulation (136, 145).

H3K4me1 marks active enhancers and persists even after H3K27ac and H3K4me3, a mark of active enhancers or promoters, is lost (146). Of note, the cooperative action of LDTFs results in cell-type specificity in which regions become epigenetically programmed upon SDTF activation (147, 148). Other histone chaperone proteins such as FACT (149), and ATP-dependent nucleosome remodelers such as SWI/SNF (human ortholog SMARCA/B) or RSC (human ortholog BAF) may catalyze unwrapping or nucleosome sliding (150, 151), thus allowing for SDTF binding (152).

Beyond chromatin modifying proteins, metabolites and alterations to metabolic flux are an integral arm of epigenetic memory (153). These mechanisms are driven by the reliance of many epigenetic modifications on metabolic processes, such as one-carbon metabolism for histone and DNA methylation (154) and generation of acetate pools from Acetyl-CoA for histone acetylation (155–157). Furthermore, mevalonate and cholesterol biosynthesis pathways are also downstream of Acetyl-CoA production, and influence the innate immune response through feedforward mechanisms at the receptor that activate PI3K signaling and mTOR (158). Increased activity of catabolic processes including glycolysis and glutaminolysis also mediate trained immunity: in human monocytes stimulated with β -glucan, glutaminolysis and cholesterol metabolism resulted in the accumulation of fumarate, which inhibited KDM5 histone demethylases to promote epigenetic reprogramming (159). Fumarate treatment of monocytes itself also mimicked β -glucan treatment by increasing both H3K4me3 and H3K27ac deposition. In human macrophages exposed to IL4, alpha-ketoglutarate, a TCA cycle intermediate, promoted demethylation of H3K27me3 in a manner dependent on Jmjd3, a histone demethylase (160). Therefore, rewiring of metabolic circuits is a key component for initiating and sustaining immune memory conveyed through histone modifications (161, 162) (Figure 4c).

3.2. Memory of prior infection

Stimulus Memory of past exposures serve the physiological purpose of changing future gene expression responses. Two main categories of innate immune memory, tolerance and trained immunity, are generated by different stimuli and alter responses in opposing directions (163). Tolerance was first described in mice surviving a lethal dose of endotoxin after having received a sublethal dose (164). It was also observed in macrophages exposed to a primary stimulus of high concentrations of lipopolysaccharide (LPS), and after a washout of up to five days, stimulated again with a secondary stimulus (165). The resulting blunted second response was accompanied by nucleosome repositioning and histone H3 lysine methyltransferase G9a, which generated heterochromatin assembly and epigenetic silencing (166). Interestingly however, tolerance is dose-dependent: when high doses of LPS, P3CSK, and poly(I:C) are diluted 100–10000 fold, hyper-responsiveness rather than tolerance may result (163). Epigenetic changes resulting in tolerance are stimulus-specific, but it remains to be seen how much the responses to heterologous secondary stimulation are also altered stimulus-specifically.

Trained immunity, involving a hyper-response of key immune regulatory genes upon stimulation, is a key outcome of stimulus-specific epigenetic memory. In response to the fungal wall component β -glucan, or *Candida albicans*, monocytes responded to

secondary stimulation with much higher production of key cytokines like TNF and IL6 (167, 168). Immune training was associated with increases in H3K4me3 and H3K4me1 (marking enhancers), even after loss of H3K27ac (marking active promoters), suggesting a stable epigenetic modification of enhancer regions help maintain trained immune memory. IFN γ secreted after initial challenge with *C. neoformans* was also shown to generate innate immune memory for up to 70 days, resulting in hyper responses of pro-inflammatory cytokines upon a secondary challenge (169). Furthermore, dendritic cells also show stimulus-specific trained immune memory. DCs treated with the fungal pathogen *Cryptococcus neoformans*, transplanted into naïve mice, and challenged again, showed increased interferon response gene expression, as well as increased production of *C. neoformans* cytokines. This apparent memory was inhibited by treatment with histone methylase inhibitors (170).

Though studies of the epigenetic plasticity and memory of innate immune responses have focused on immune cells like macrophages, there is emerging evidence that fibroblasts, stromal cells, and hematopoietic stem cells may also be pliable to stimulus-specific epigenetic programming (171). These cells have longer lifespans than circulating monocytes, and may thus be the optimal messengers to carrying memory of past exposures (172, 173). Other cell types with longer memory may include fibroblasts, as chromatin marks deposited after IFN β stimulation led to faster and increased expression of interferon genes on a second stimulation (174). Epithelial stem cells were also shown to maintain memory of a primary response through sustained increase in chromatin accessibility at key inflammatory response genes, heightening responses to subsequent inflammatory stimuli (175).

3.3. Stimulus Memory *via* vaccination

Given its role in physiology, several attempts have been made to harness Stimulus Memory and the training of innate immunity through vaccination. The tuberculosis vaccine BCG (Bacillus Calmette-Guérin) is a well-known example (176), where vaccination with this attenuated bacterium provides broad protection against multiple bacterial and fungal organisms through hyper-response of key genes upon secondary stimulation (177). BCG-trained immunity not only affected monocytes via H3K27ac histone modifications (178), but also impacted the epigenetic landscape of hematopoietic stem cells (179, 180). BCG-trained HSCs led to epigenetically modified monocytes and macrophages that had alterations in H3K4me1, H3K4me3, and H3K27ac, and cleared tuberculosis infections more effectively than naïve macrophages (180). Trained immunity of progenitor cells like HSCs may explain the lasting effects of innate immune vaccination. Importantly, while programming the epigenetic landscape is specific to the stimulus, unlike vaccines targeted at adaptive immunity that aim to generate memory B-cells, innate immune vaccination by BCG provides heterologous effects and protects individuals from many other bacterial, viral, or fungal pathogen threats (181, 182).

Tolerance and immune training *via* treatment with LPS or BCG, respectively, has also been suggested as a potential avenue for the modulation of autoimmune diseases like systemic sclerosis (183), which is marked by fibrosis as a result of chronic but ‘sterile’ inflammation

(184). Treatment of macrophages with low-dose LPS generated a tolerized phenotype that reduced inflammation-related fibrosis in a mouse model. On the other hand, BCG exposure generated a trained phenotype with increased production of pro-inflammatory cytokines, exacerbating the fibrotic process. LPS and BCG generated unique epigenomic changes, with gene-specific changes in chromatin marks, including H3K4me3 (183).

3.4. Diseases of dysregulated Stimulus Memory

Severe pathology can result from dysregulated immune memory. Sepsis, which involves hyperactivation of immune response as well as immune paralysis that prevents the clearance of bacteria in the bloodstream, affects millions of people yearly and nearly 1/3 of hospital deaths are caused by sepsis. Both tolerance and trained immunity play roles. Tolerance serves to eliminate hyper-response on secondary stimulation, but misapplied regulation of tolerance results in poor host defense to secondary exposures to bacterial stimuli. Interestingly, the metabolic output of TCA cycle decarboxylation, itaconate, promoted tolerance in human monocytes, while β -glucan inhibited IRG1, the enzyme that promotes itaconate synthesis, leading instead to an enhanced secondary immune response (185). The ability of specific stimuli to generate trained immunity and revert disease-causing tolerance could lead to additional methods to modulate the immune system during or after infection.

Another disease of dysregulated immune memory is hyper IgD syndrome (HIDS), an inborn error of metabolism where mevalonate kinase deficiency leads to accumulation of mevalonate (186). Monocytes and macrophages in these patients produce higher amounts of TNF, IL6, and IL1b, and anti-TNF and anti-IL1 therapies have only been partially effective (187). The metabolite mevalonate was shown to be critical in β -glucan- and oxLDL-induced trained immunity by driving the mTOR pathway, activating the TCA cycle, and generating acetyl-CoA needed for altered H3K27ac at inflammatory genes (188). The chronically trained immunity state of macrophages due to elevated mevalonate may be a cause of the 'sterile' inflammatory phenotype seen in these patients, such as febrile attacks, arthritis, and skin lesions (188). Importantly, administration of statins blocked the mevalonate-cholesterol synthesis pathway, attenuating trained immunity and reducing inflammatory attacks (189).

Outlook

The physiological roles of macrophages are many(190, 191), and they are precisely regulated. Here we have proposed that these varied physiological roles depend on a key set of three functional hallmarks. For each of these hallmarks, addressing outstanding questions will bring us closer to harnessing and controlling macrophage function, either for diagnostics or for the treatment of disease. We discuss a few open questions below and outline further potential lines of inquiry in the Future Issues box.

Might Response Specificity inform us about risk for inflammatory disease? Response Specificity, a property of macrophages that is affected by both cytokine context and immune memory, may prove a convenient metric for measuring health and disease states. In multiple immune diseases, noisy or ineffective recognition of an inflammatory threat lead to autoimmunity or faulty pathogen clearance. The functional health of the innate immune system, which is affected by context or prior exposures, could in the future

be measured by perturbing monocytes isolated from peripheral blood, and profiling the resulting transcriptome or epigenome. The diagnosis or prognosis for a wide variety of diseases depends on immune system function, including autoimmune diseases, cancers, or neurodegenerative diseases, to name a few. It remains to be seen to what extent monocyte and macrophage Response Specificity is reflective of risk or disease stage of each of these.

Will understanding Context Dependence allow us to predictably tune macrophage responses with microenvironmental cues? A central difficulty in understanding Context Dependence has been to clarify the effect of different ligands or ligand combinations, their doses, their duration, or temporal order of exposure. *In vitro* studies have isolated the effects of particular combinations of contextual cytokines, often recapitulating Context Dependence responses that arise by exposure to real pathogens or from inflammatory diseases. Further work in uncovering the signaling and epigenetic mechanisms that channel macrophage responses, may in the future allow us to use specific adjuvants to manipulate favorable microenvironment conditions for cancer, atherosclerosis, or metabolic disorders.

Might we harness Stimulus Memory to strengthen innate immunity to improve human health? A recent study reported that four-weeks of aerobic exercise prior to surgery created a lasting phenotype of immune tolerance in Kupffer cells, improving ischemia-reperfusion injury outcomes (192). However, further study is needed to understand the physiological consequences of training innate immunity. For example, innate immune memory may play roles in post-COVID19 immunity or inflammatory sequelae. A recent study on convalescing COVID19 patients indicated altered monocyte subsets after COVID, with increased chromatin accessibility at inflammatory genes in patients covering from COVID19, suggestive of trained immunity. CD14+ and CD16+ monocytes from convalescing patients maintained epigenetic modifications and had increased IL6 and IL-1 β production on subsequent stimulation with spike-nCoV pseudovirus (193). It remains to be determined whether this trained immunity results in an effect similar to vaccination, protecting the individual from subsequent infection, or whether the subsequent hyperinflammatory responses predisposes individuals to syndromes of long-COVID.

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Terms and Definitions

Sentinel cell	a cell that conveys messages about the environment to activate other cells
PAMP	pathogen-associated molecular pattern; pathogen-derived molecules recognized by macrophage receptors
DAMP	damage-associated molecular pattern; host-cell derived molecules indicative of damage like DNA, histones, ATP, and other nuclear or cytosolic proteins

PRR	pattern recognition receptor; sensors that detect molecules related to pathogens (PAMPs) or cell damage (DAMPs)
Polarization	the ability of macrophage to adopt specialized functions as an adaptation to contextual cytokines or other microenvironmental signals
M1/M2 macrophages	two extremes of macrophage polarization, often generated by IFN γ or IL4 cytokine contexts, respectively
Priming	microenvironment context sets up the epigenetic landscape for stronger induction upon PAMP, DAMP, or cytokine stimuli, without return to baseline
SDTF	signal-dependent transcription factor; transcription factors activated upon an extracellular stress signal
LDTF	lineage-determining transcription factor; transcription factors that establish cell type by remodeling the enhancer landscape
Pioneer factor	a transcription factor that contains a DNA anchoring α helix that allows direct binding to nucleosomal DNA
Tolerization	retainment of epigenetic histone modifications after a first exposure that suppresses the response to a second
Trained immunity	retainment of epigenetic histone modifications after a first exposure that generates hyper-inflammatory response to a second

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Summary Points

The health of the immune system depends on the health of macrophage function, as they are the orchestrators of immune activation. Three functional hallmarks mark healthy macrophage responses: Response Specificity, Context Dependence, Stimulus Memory.

Response Specificity is the ability to single macrophage cells to selectively activate particular gene programs appropriate to the pathogen, and is evaluated by overlap of single cell ligand-response distributions.

Context Dependence refers to the canalization of macrophage responses relative to polarizing cytokines or signals from the microenvironment.

Stimulus Memory is the ability of macrophages to store specific marks of prior exposures stably within the epigenome, altering future responses.

Immunological diseases involving macrophage responses arise from combinatorial dysregulation of these functional properties. Understanding mechanisms that generate these functions will allow us to measure them for diagnosis or manipulate them for treatment.

Future Issues

As regulators of Response Specificity, what mechanisms are the key sources of cell-to-cell heterogeneity and do they cause pathology?

What features of Response Specificity are predictive of innate immune health and which are impaired when immune health is compromised by so-called 'pre-existing conditions'?

Context Dependence is regulated by polarizing cytokines, but what are the other microenvironmental components such as nutrients?

In understanding Context Dependence mechanisms operating on signaling and epigenetic networks may we be able to develop predictive interventions to improve macrophage function?

In encoding Stimulus Memory, what cofactors assist immune response transcription factors in evicting nucleosomes and establishing *de novo* enhancers?

Is Stimulus Memory sufficiently long and reliable such that trained immune precursor cells may produce trained differentiated macrophages that can provide health benefits?

Sidebar element

In comparison to macrophages, other cell types can exhibit either effector or sentinel functions (7). Fibroblasts, which form part of the connective tissue, are tissue-resident sentinels, similarly express PAMP, DAMP, and cytokine receptors, and activate stimulus-specific immune response genes upon ligand challenge (8–11). Endothelial cells, placed in a prime position to respond to circulating endotoxins (12, 13), are also sentinels that produce inflammatory cytokines to mobilize other immune cells (14–16). However, these structural sentinel cells do not exhibit the strong effector functions of macrophages. Conversely, neutrophils and NK cells are not tissue-resident and do not serve in primary roles as stimulus-specific sentinels. However, they each share some of the effector functions of macrophages such as phagocytosis or release of anti-pathogen lytic enzymes, respectively (17–19).

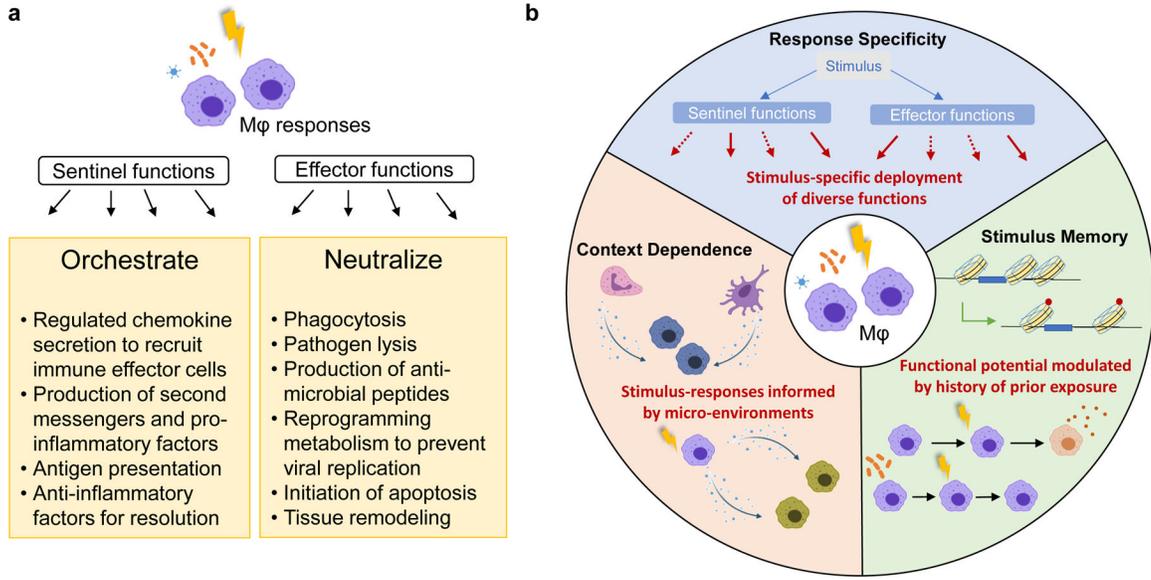


Figure 1. Functional hallmarks of macrophages. (a) Macrophages respond by performing a variety of functions. Categorization of these functions show that macrophages can perform as sentinel cells of the immune system or as immune effector cells. (b) Macrophage responses exhibit three hallmarks central to immunological function: Response Specificity, Context Dependence, and Stimulus Memory. Dashed and solid arrows represent deployment of specific functions to different degrees or speeds.

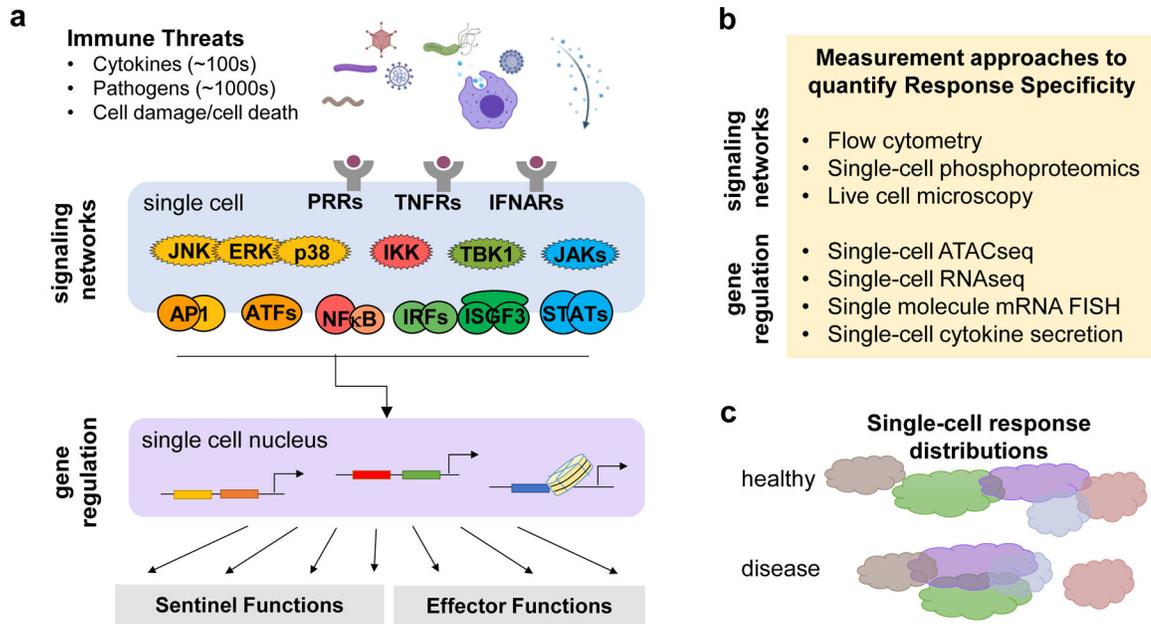


Figure 2.

Mechanisms and measurement approaches of Response Specificity. (a) Response Specificity relies on the ability of dozens of pathogen recognition receptors and cytokine receptors to recognize specific ligands. Ligand-receptor interactions activate specific signaling pathways with ligand-specific temporal and dose dynamics, which are recognized by gene regulatory mechanisms that decode the stimulus-specific combinations of temporally modulated transcription factor activities. Single cell heterogeneity in signaling network activation and transcriptional regulation all impact Response Specificity. (b) Single-cell measurements of signaling or epigenetic events that can be interrogated to quantify Response Specificity, (c) resulting in an understanding of ligand-response distributions in health versus disease. Abbreviations: ATAC-seq, assay for transposase-accessible chromatin with sequencing; FISH, fluorescence in situ hybridization; RNA-seq, RNA sequencing; PRR, pattern recognition receptor; TNFR, Tumor necrosis factor receptor; IFNAR, interferon alpha/beta receptor; AP1, activator protein 1; ATFs, Activating transcription factors; NFκB, nuclear factor kappa B; IRFs, interferon response factors; ISGF3, interferon stimulated gene factor 3; STATs, signal transducer and activator of transcription.

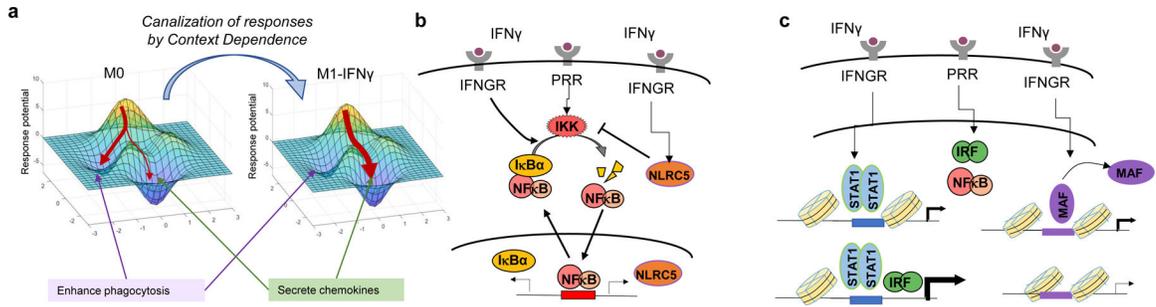


Figure 3. Context Dependence is mediated by microenvironmental signals that may lead to a canalization of the diverse stimulus-specific macrophage responses. (a) Cytokine context results in specialization of function by reversibly altering the epigenetic states of signaling and gene regulatory networks. Arrows pointing to regions of the response landscape represent possible responses given an inflammatory stimulus. (b) Positive and negative regulation of signaling feedback regulators by polarizing cytokines may generate context-dependent signaling profiles. (c) Epigenetic mechanisms that either hold open promoters or disassemble enhancers allow for gene-specific regulation of context-dependent responses in macrophages. Abbreviations: PRR, pattern recognition receptor; IFNGR, interferon gamma receptor.

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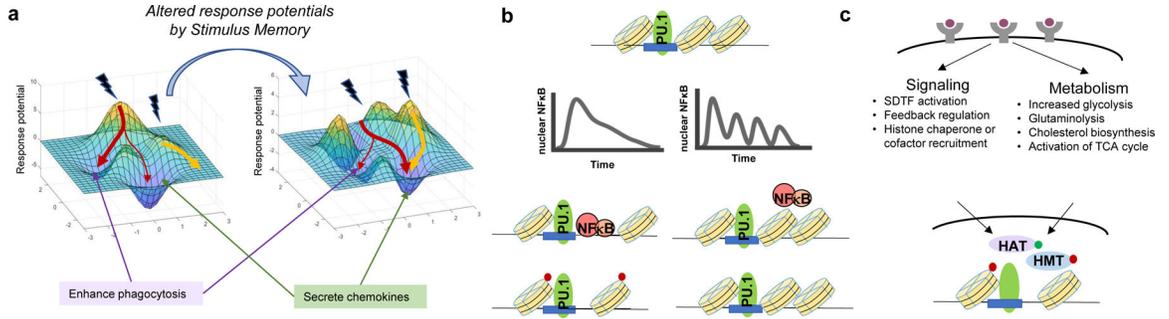


Figure 4. Stimulus Memory involves prior exposure altering epigenetic states of signaling and gene regulatory networks. (a) Stimulus Memory is mediated for example by changes to the chromatin enhancer landscape, altering response potential after the initial stimulus has subsided. Arrows pointing to regions of the response landscape represent possible responses given an inflammatory stimulus. (b) Stimulus-specific non-oscillatory activity of SDTFs opens chromatin in collaboration with cofactors and chromatin-remodeling enzymes. (c) Both signaling pathway activation and alterations to metabolic pathway activity are critical arms for generating innate immune memory. Abbreviations: HAT, histone acetyltransferase; HMT, histone methyltransferase; SDTF, signal-dependent transcription factor; TCA, tricarboxylic acid; NFκB, nuclear factor kappa B; PU.1, PU-box binding factor.

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